### STIC-ILL

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1. Bau et al, NAR 22:2811-2816 (1994)

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Thanks, Melissa 11D05

11th floor mailbox

position -35; an Alu I-Barn HI fragment from pML(C<sub>2</sub>AT) (9), consisting of 377 bp of G-minus sequence, followed by 5 bp of Sma I and Barn HI recognition sequences. The UAS fragment in pGAL4CG— was derived from pCZGAL (15) and contained a single GAL4-binding site. The UAS fragment in p(DED48)<sup>2</sup>CG— was derived from pCZ(DED48)<sup>2</sup> (A. R. Fuchman and R. D. Kornberg, in preparation) and contained two copies of the T-rich element from upstream of the DED1 the T-rich element from upstream of the DED1 gene. Removal of the GAL4-binding site from pGAL4CG- by cleavage with Kba I and Eco RI, followed by filling in with the large fragment of DNA polymerase I and ligation, gave pΔCG-.
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 We thank A. Edwards and R. Kelleher for help in the control of the

developing the assay for initiation in yeast extracts with G-minus templates. We thank R. Roeder for gifts of pML(C<sub>2</sub>AT) and p(C<sub>2</sub>AT)19, and R. Tjian for a gift of HeLa nuclear extract. K.S. received partial support from the Toyobo Biotechnology Foundation. Costs of this research were paid from NIH grant GM36659 (R.D.K.).

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#### Fig. 1. Detection of protein-RNA complexes by band-shift assay. Cytoplasmic extracts of Jurkat cells were prepared by freeze-thaw lysis in 25 mM tris-HCl (pH 7.9), 0.5 mM EDTA, and 0.1 phenylmethylsulfonyl fluoride, followed centrifugation at 15,000g at 4°C for 15 min. RNAs were transcribed by T7 RNA



polymerase and labeled with [32P]UTP (uridine triphosphate) to a specific activity of 107 cpm per microgram of RNA from Eco RI-digested pT7/T3-α19 (60-base nonspecific probe) (BRL) or Kpn I-digested pT7/T3-α19-AUJIIIA [64base specific probe with four AUUUA repeats (16); coding oligonucleotides were cloned into the unique Sma I site]. Cytoplasmic extract (from  $2 \times 10^{3}$  cells) was incubated with  $10^{4}$  cpm of RNA (0.5 to 1 ng), in 10% glycerol, 12 mM Hepes (pH 7.9), 15 mM KCl, 0.25 mM EDTA, 0.25 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, and Escherichia coli transfer RNA (200 ng/µl) in a total volume of 10 µl for 10 min at 30°C. RNase A was added to a final concentration of 1  $\mu g/\mu l$ , and reaction mixtures were incubated for 30 min at 37°C before electrophoresis in a 7% native polyacrylamide gel with 0.25× TBE running buffer (10). Lane I, AUUUA-containing specific probe alone; lane 2, specific probe and lysate; lane 3, nonspecific probe alone; and lane 4, nonspecific probe and lysate. The positions of complexed and free probe are indicated.

tained four adjacent reiterations of the AUUUA morif After a brief incubati n, the reaction mix was treated with ribonuclease A (RNase A) and the presence of protected complexes then assessed by band-shift assay on native, low ionic strength polyacrylamide gels (Fig. 1). A stable, RNase A-resistant complex was detected. Neither a control RNA probe of similar size, nucleotide content, and specific activity but lacking the AUUUA reiterations (Fig. 1) nor an RNA probe of similar size containing four UAAAU repeats formed stable complexes (9). The complex formed with the probe containing the AUUUA motifs was stable in the presence of RNase A for at least 2 hours (9) or when resolved in higher ionic strength running buffers such as  $0.5 \times$  or  $1 \times$ tris-borate-EDTA (TBE) (9, 10). Complex formation was abolished by prior incubation of the lysate with proteinase K (2.5 mg/ml) for 15 min (9).

The molecular size of the complex was assessed after ultraviolet (UV) light-induced cross-linking of the lysate-probe reaction mixture and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). A stable complex migrating with a molecular mass of 28 to 45 kD was observed with the AUIUIA-containing probe, but not the control probe, and stable complexes were not bserved when specific or nonspecific

## Identification of an AUUUA-Specific Messenger **RNA Binding Protein**

James S. Malter

An important control point in gene expression is at the level of messenger RNA (mRNA) stability. The mRNAs of certain regulatory cellular proteins such as oncogenes, cytokines, lymphokines, and transcriptional activators are extremely labile. These messages share a common AUUUA pentamer in their 3' untranslated region, which confers cytoplasmic instability. A cytosolic protein was identified that binds specifically to RNA molecules containing four reiterations of the AUUUA structural element. This protein consists of three subunits and binds rapidly to AUUUAcontaining RNA. Such protein-RNA complexes are resistant to the actions of denaturing and reducing agents, demonstrating very stable binding. The time course, stability, and specificity of the protein-AUUUA interaction suggests the possibility that the formation of this complex may target susceptible mRNA for rapid cytoplasmic degradation.

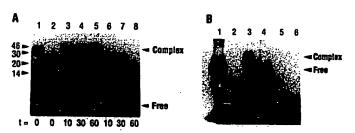
ESPITE GREAT PROGRESS IN ELUcidating the mechanisms of transcriptional regulation of gene expression, relatively little is known about post-transcriptional control at the level of mRNA turnover (1). In a wide variety of organisms and cell types, mRNAs display heterogeneous cytoplasmic stability (1). Inducible growth regulators such as oncogene products (2), cytokines (3), and transcriptional activators (4) tend to have extremely unstable messages with half-lives on the order of 10 to 30 min. Treatment of cells with phorbol esters (5), antibodies to cell surface proteins (6), serum (4), or protein synthesis inhibitors such as cycloheximide (6, 7) can modulate the half-lives of rapidly degraded messages. Therefore, the rates and selectivity f mRNA degradati n are variable, and regulation of these processes are important control points of gene expression.

The mechanisms by which mRNA is targeted for rapid turnover are poorly understood (1). A recurrent motif of rapidly degraded mRNA is an AU-rich structure in the 3' untranslated region (1, 8). In particular, the pentamer AUUUA is present singly or in multiple reiterations in a wide variety of oncogene and cytokine mRNA 3' untranslated regions. Removal of this region confers significantly greater stability to messages produced from transfected constructs (2, 7, 8), whereas the addition of a short DNA segment coding for this motif destabilized previously stable messages (8). Thus it has been proposed that a trans factor may recognize this AU-rich motif and in some way target susceptible mRNA for degradation (1, 8).

To determine if a cytoplasmic protein ( r proteins) specifically interacts with the 3' untranslated region of unstable mRNA through the AUIUIA element, we incubated lymphocyte cytopiasmic extract with in vitro transcribed, labeled RNA that con-

Department of Pathology, Tulane University School of Medicine, New Orleans, LA 70112.

Fig. 2. Stability of the protein-RNA complex, with or without cross-linking, after SDS-PAGE. Reaction mixtures were prepared and incubated as described in Fig. 1, treated or not treated with RNase A, and either exposed to UV



light (17) [for the length of time (t) in minutes, as indicated at bottom of (A)] or processed immediately (B) by boiling with an equal volume of 2× SDS-sample buffer (18) before SDS-PAGE. (A) Lane 1, specific probe and lysate, with RNase A treatment, and without UV light; lanes 3 to 5, specific probe and lysate with RNase A treatment, and with UV light; and lanes 6 to 8, nonspecific probe and lysate, with RNase A treatment, and with UV light; and lanes 6 to 8, nonspecific probe and lysate, with RNase A treatment, and with UV light. Molecular sizes (in kilodaltons) are shown on the left. (B) Lane 1, specific probe, without RNase A treatment; lane 2, specific probe, with RNase A treatment; lane 3, specific probe and lysate, with RNase A treatment; lane 4, nonspecific probe, without RNase treatment; lane 5, nonspecific probe, with RNase A treatment; lane 6, nonspecific probe and lysate, with RNase A treatment; lane 6, nonspecific probe and lysate, with RNase A treatment.

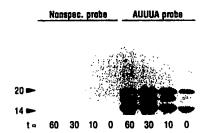


Fig. 3. The effect of denaturation and disulfide bond reduction on stability of the protein-RNA complex with or without UV cross-linking. Reaction mixtures were prepared and incubated as described in Fig. 1, but boiled in an equal volume of 2× SDS-sample buffer containing 5% 2-mercaptoethanol before SDS-PAGE. Time of exposure to UV light (t) is in minutes; nonspec., nonspecific. Molecular sizes (in kilodaltons) are shown on the left.

probes and RNase A were incubated alone (Fig. 2B). Unexpectedly, stable complexes were consistently found in reaction mixtures immediately subjected to electrophoresis without exposure to UV light (Fig. 2B), suggesting that the UV treatment was not required for complex stability.

Reduction of the lysate-probe mixture with 2-mercaptoethanol followed by SDS-PAGE resolved the complex into three components of approximately 15, 17, and 19 kD (Fig. 3). These data suggest that each of the protein subunits has a binding site for the AUUUA sequence. Because dissociation of the binding protein [herein denoted adenosine-uridine binding factor (AUBF)] occurred after treatment with reducing agents, the subunits are likely held together by interchain disulfide linkages. The stability of the complex under denaturing or reducing conditions is presumably the result of highly specific, hydrogen-bond interacti ns between AUBF and RNA. My observati ns are consistent with the high-affinity RNAbinding characteristics of a family of heterogeneous nuclear ribonucleoproteins (11). It

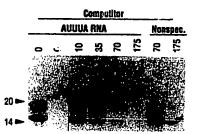
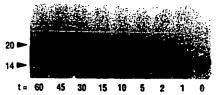


Fig. 4. Inhibition of formation of the RNA-protein complex by competitor RNA. Reaction mixtures were as described in Fig. 1, except that various amounts of unlabeled specific or nonspecific RNA were incubated with the cytoplasmic lysate for 10 min at 30°C before the addition of labeled, specific probe for 10 min. After RNase A treatment for 30 min, all samples were exposed to UV light for 20 min at 0°C and boiled in 2× SDS-sample buffer containing 5% 2-mercapto-chanol. Lane 1, specific probe and lysate, without unlabeled competitor; lanes 3 to 8, specific probe and lysate in the presence of increasing molar excess (shown) of unlabeled AUUUA RNA or control, nonspecific RNA.

is not known whether the subunits represent dissimilar or related peptides, as variable nuclease protection of the probe by individual subunits could account for the small observed differences in complex molecular mass (12).

The specificity of the AUBF-RNA interaction was assessed by competition experiments. Lysate was incubated with increasing amounts of unlabeled AUUUA-containing probe or control RNA before incubation with labeled AUUUA-containing probe (Fig. 4). The unlabeled specific probe effectively competed with its labeled analog for binding to the three protein subunits. A 100-fold excess of homoribonucleotide polymers [poly(A) r poly(U)] or coribonucleotide polymers [poly(A, U), poly(A, C, U), or poly(A, G, U)] did not significantly decrease specific probe binding (9), indicat-



Flg. 5. Binding kinetics of RNA-protein complex. Reaction mixtures were as described in Figs. 1 to 3, except that specific probe and lysate were incubated at 30°C for the times (t) (in minutes) shown before the addition of RNase A. After 30 min at 37°C, samples were boiled in 2× SDS-sample buffer containing 5% 2-mercaptoethanol before SDS-PAGE.

ing AUBP does not effectively recognize poly U tracts.

To assess the kinetics of interaction between the AUUUA-containing probe and cytoplasmic binding activity, I incubated the probe with lysate for various times before the addition of RNase A (Fig. 5). Incubations as short as 1 min yielded the same amount of complex as 1-hour incubations. When lysate, probe, and RNase A were added simultaneously, all three subunits were detected, although with decreased intensity (Fig. 5). Thus, stable complex formation appears to occur rapidly and likely precedes exonuclease-mediated shortening of polyadenylated tails that appears to be the primary degradative event of labile mRNA (7, 13).

The number of reiterations or which regions of the AUUUA pentamer are required for the stable, specific binding observed here is not known, nor is molecular mechanism of AUBF-RNA interaction. Active site sulfhydryls of the iron response element-binding protein have been implicated as the mediators of binding to the iron response element in ferritin mRNA (14). The stability of the AUBF-RNA complex is consistent with such a mechanism. In addition, because the mRNA degradative system can discriminate between different AU-rich mRNAs (15), it is possible that AUBF is a member of a family of proteins with multiple domains for recognition of the common AUUUA motif as well as other message-specific ele-

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 Experiments occasionally demonstrated a distinct 14-kD species (compare Fig. 3 to Fig. 5) after sample preparation with 2-mercaptoethanol. This species usually appeared as a smear, suggesting it arose from variable cleavage by RNase A of the RNA probe annealed to the 15-kD subunit.

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16. The specific probe contained the T7 start site at the 5' end linked by a polylinker to the sequence 5'-GGAUCCAUUUAUUUAUUUAUUUAAGC-UUGG3'.

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# Prevention of Allogeneic Bone Marrow Graft Rejection by H-2 Transgene in Donor Mice

Claes Ohlén, Gunilla Kling, Petter Höglund, Mona Hansson, George Scangos, Charles Bieberich, Gilbert Jay, Klas Kärre\*

Rejection of bone marrow grafts in irradiated mice is mediated by natural killer (NK) cells and is controlled by genes linked to the major histocompatibility complex (MHC). It has, however, not been possible to identify the genes or their products. An MHC class I (D<sup>d</sup>) transgene introduced in C57BL donors prevented the rejection of their bone marrow by NK cells in irradiated allogeneic and F1 hybrid mice expressing the D<sup>d</sup> gene. Conversely, H-2D<sup>d</sup> transgenic C57BL recipients acquired the ability to reject bone marrow from C57BL donors but not from H-2Dd transgenic C57BL donors. These results provide formal evidence that NK cells are part of a system capable of rejecting cells because they lack normal genes of the host type, in contrast to T cells, which recognize cells that contain abnormal or novel sequences of non-host type.

CCEPTANCE AND REJECTION OF skin grafts can be predicted from the transplantation laws, reflecting the control of the T cell repertoire by genes for histocompatibility antigens (1). Only grafts expressing genes for antigens that are not expressed in the recipient can be recognized and eliminated by T cells. MHC-disparate bone marrow grafts can also be rejected by a T cell-independent mechanism involving NK cells (2, 3). With classical genetic crosses, it has not been possible to determine whether this type of rejection is triggered by expression of nonrecipient H-2, expression of foreign products encoded by other than the known MHC class I or class II genes, or absence of recipient H-2. We used trans-

genic mice to distinguish between these alternatives. Apart from their implications for basic immunology, the rules for rapid NK-mediated elimination of MHC-disparate hemopoietic cells are important for research on bone marrow transplantations, graft versus host reactions and viral diseases where transmission between individuals may occur by transfer of infected blood cells

Transgenic mice of the D8 founder strain were produced by microinjection of an 8.0kb genomic clone encoding H-2Dd and flanking regions from BALB/c into a C57BL/6 (B6) zygote (5). The cell surface expression of the H-2D<sup>d</sup> transgene product showed the same tissue distribution and levels as the endogenous Kb and Db products (5). A total of 321 mice of different genotypes received 900 rad of whole-body irradiation, and were then given either bone marrow from B6 or transgenic D8 mice, or no cells at all. Bone marrow engraftment was monitored by splenic 125 I-labeled iododeoxyuridine (125IUdR) uptake on day 5. With this assay, it has been shown that B6 bone marrow is rejected by NK cells in H-2Dd-expressing B10.D2 and (B10.D2 × B6) $F_1$  mice (2, 3) (Fig. 1). If rejection was

due to absence of H-2D<sup>d</sup> products in the B6 marrow graft (6, 7), H-2Dd transgene expression in the donor should lead to acceptance, as was indeed observed (Fig. 1). The H-2Dd transgene had no effect on rejection of B6 marrow in B10.BR, an MHC disparate strain that does not carry the H-2D<sup>d</sup> gene (Fig. 1).

These results are consistent with a model in which the transgene conferred protection selectively against a response, controlled by H-2Dd at the host level, that is geared to detect the absence of the corresponding gene or product in the graft (6, 7). According to this model, when D8 mice are recipients, expression of the H-2D<sup>d</sup> transgene should be sufficient to render them capable of rejecting B6 marrow (even if they do not reject B6 skin grafts) (8, 9). Homozygous D8 as well as  $(D8 \times B6)F_1$  mice rejected B6 grafts, although the rejection in the F1 hybrid appeared weaker (Fig. 1). H-2Dd transgenic bone marrow was accepted in both recipients.

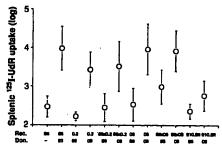


Fig. 1. Effect of H-2Dd transgene on engraftment of bone marrow in irradiated recipients (Rec.), monitored by <sup>125</sup>IUdR uptake in spleen. Bone marrow from tibia and femur was obtained by flushing with phosphate-buffered saline as described (2). One million bone marrow cells were grafted from B6 (H-2b) and H-2Dd transgenic D8 donors (Don.) by intravenous inoculation to irradiated (900 rad) recipients of different genotypes. On day 5, 3 µCi of 125 IUdR (Amersham) was inoculated into mice intraperitoneally, and 18 to 24 hours later, the animals were killed and the radioactivity in the spleens was measured in an LKB gamma counter. After subtraction of background, log<sub>10</sub> values were calculated. The geometric means and standard deviations in each group were calculated from several pooled experiments, each of which included several recipient genotypes in parallel. Each genotype was tested in two to four experiments, always with B6 and D8 grafts in parallel. Irradiated, nongrafted B6 mice and B6 mice grafted with syngeneic bone marrow were included in all experiments as controls. The incorporation of <sup>125</sup>IUdR in irradiated mice without grafts was similar in all genotypes and is shown here for B6 mice only. The difference between B6 and D8 donors was evaluated for each recipient by the Student's t test. (P < 0.001in all cases, except in B10.BR recipients where the difference was nonsignificant.) D8, B6 (H-2b), DBA2 (H-2<sup>d</sup>), and F<sub>1</sub> hybrids were bred and maintained at the Department of Tumor Biology. B10.D2 (D2, H-2<sup>d</sup>) and B10.BR (H-2<sup>k</sup>) were purchased from the Jackson Laboratory.

Sweden.
G. Scangos, Molecular Therapeutics, Inc., West Haven, CT 16516.
C. Bieberich, Department of Biology, Yale University, New Haven, CT 06510.
G. Jay, Virology Laboratory, Jerome H. Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville. MD 20835. Rockville, M.D. 20855.

C. Öhlén, P. Höglund, K. Kärre, Department of Tumor Biology, Karolinska Institute, Box 60400, S-104 01 rockholm, Sweden.

J. Kling and M. Hansson, Department of Immunology, Karolinska Institute, Box 60400, S-104 01 Stockholm,

<sup>&</sup>quot;To whom correspondence should be addressed.